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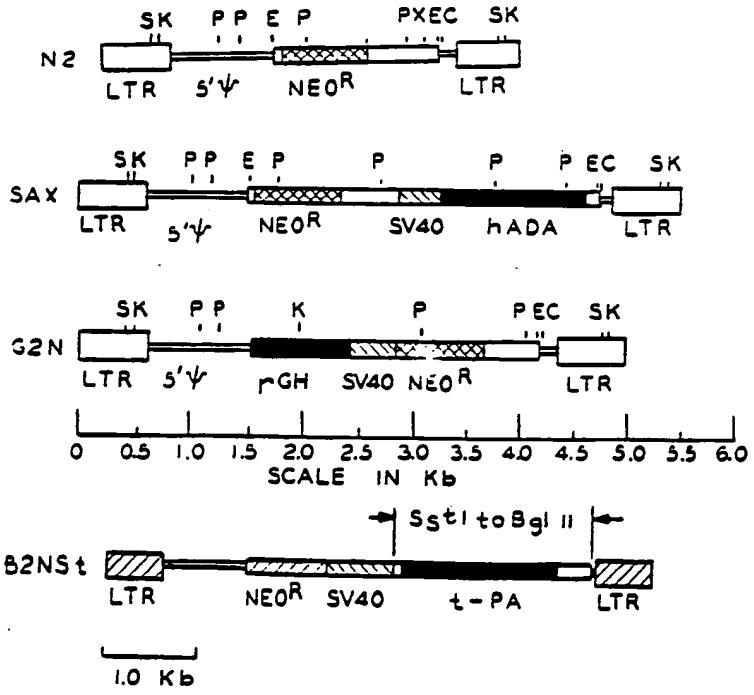

(57) Abstract

Endothelial cells are genetically engineered with a gene for a heterologous protein which is a therapeutic agent. The endothelial celles may be seeded onto a vascular graft and implanted in the vascular system of a mammal to produce the therapeutic agent *in vivo*.

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GENETICALLY ENGINEERED ENDOTHELIAL CELLS AND USE THEREOF

This invention relates to genetically engineered cells, and to the use thereof. Still more particularly, this invention relates to genetically engineered endothelial cells and the use thereof for expressing a therapeutic agent.

There have been numerous proposals with respect to genetically engineering mammalian cells. In general, retroviruses have been employed for introducing genetic material into mammalian cells. Thus, there have been proposals to genetically engineer bone marrow and hematopoietic progenitor cells by the use of retroviral vectors. In general, there have been drawbacks to the use of such cells, such as the variable ability to express certain genes and/or inefficient gene transfer. Thus, there have been further proposals for genetically engineering cells capable of both long term survival and stable expression including cells such as fibroblasts, lymphocytes, keratinocytes and hepatocytes for use in gene therapy.

The present invention is directed to genetically engineered endothelial cells, and the use thereof for expressing a heterologous protein. In one embodiment the heterologous protein is a therapeutic agent.

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According to one aspect of the present invention, there is provided endothelial cells which are transformed with at least one gene which encodes for at least one heterologous protein, which is preferably a therapeutic agent.

In accordance with another aspect of the present invention, there is provided a solid support which includes endothelial cells transformed with at least one gene which encodes for at least one heterologous protein, preferably a therapeutic agent. In a preferred embodiment, the solid support is one which is compatible with blood and may, for example, be in the form of a blood vessel graft.

In accordance with yet another aspect of the present invention, endothelial cells which are transformed with at least one gene which encodes for at least one heterologous protein, preferably a therapeutic agent are implanted in a blood vessel.

More particularly, the endothelial cells employed in the present invention are endothelial cells derived from a mammal. The endothelial cells are obtained from a blood vessel. The term "blood vessel" as used herein includes veins, arteries and capillaries. Thus, the endothelial cells which are genetically engineered include macrovascular and/or microvascular endothelial cells.

The mammalian cells may be derived from a human or nonhuman mammal. The endothelial cells are preferably derived from a human.

The endothelial cells are transformed with at least one gene which encodes for at least one heterologous protein which is preferably a therapeutic agent. The cells may be transformed in a manner in which the therapeutic agent is secreted from the transformed cells or may be transformed in a manner in which the therapeutic agent remains in or on the transformed cells.

The mammalian endothelial cells are transformed with a suitable vector or expression vehicle which includes a gene for at least one therapeutic agent. The vector includes a promoter

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for expression in mammalian cells; for example, SV 40, LTR, metallothionein, PGK; CMV; ADA; TK; etc. The vector may also include a suitable signal sequence or sequences for secreting the therapeutic agent from the cells. The selection of a suitable promoter is deemed to be within the skill of the art from the teachings herein.

The expression vehicle or vector is preferably a viral vector and in particular a retroviral vector. As representative examples of suitable viral vectors which can be modified to include a gene for a therapeutic agent, there may be mentioned: Harvey Sarcoma virus; ROUS Sarcoma virus, MPSV, Moloney murine leukemia virus, DNA viruses (adenovirus) etc. Alternatively, the expression vehicle may be in the form of a plasmid. The expression vehicle may also be in a form other than a vector; for example, transformation may be accomplished by liposome fusion, calcium phosphate or dextran sulfate transfection; electroporation, lipofection, tungsten particles etc. The selection of a suitable vehicle for transformation is deemed to be within the scope of those skilled in the art from the teachings herein.

In employing a retroviral vector as the expression vehicle for transforming endothelial cells, steps should be taken to eliminate and/or minimize the chances for replication of the virus. Various procedures are known in the art for providing helper cells which produce viral vector particles which are essentially free of replicating virus. Thus, for example, Markowitz, et al., "A Safe Packaging Line for Gene Transfer: Separating Viral Genes on Two Different Plasmids," Journal of Virology, Vol. 62, No. 4, pgs. 1120-1124 (April 1988); Watanabe, et al., "Construction of a Helper Cell Line for Avian Reticuloendotheliosis Virus Cloning Vectors, "Molecular and Cellular Biology, Vol. 3, No. 12, pgs. 2241-2249 (Dec. 1983); Danos, et al., "Safe and Efficient Generation of Recombinant Retroviruses with Amphotropic and Ecotropic Host Ranges, "Proc.

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Natl. Acad. Sci. Vol. 85, pgs. 6460-6464 (Sept. 1988); and Bosselman, et al., "Replication-Defective Chimeric Helper Proviruses and Factors Affecting Generation of Competent Virus: Expression of Moloney Murine Leukemia Virus Structural Genes via the Metallothionein Promoter, "Molecular and Cellular Biology, Vol. 7, No. 5, pgs. 1797-1806 (May 1987) disclose procedures for producing a helper cell which minimizes the chances for producing a viral particle which includes replicating virus. This procedure and other procedures may be employed for genetically engineering with endothelial cells by use of a retroviral vector.

The endothelial cells which are to be genetically engineered in accordance with the present invention may be derived from a mammal, and as hereinabove indicated, such endothelial cells may be obtained from an appropriate blood vessel, such as an artery, vein or capillary. The procedure for obtaining such endothelial cells from the blood vessel of a mammal are generally known in the art and a representative procedure is disclosed in the Examples.

The invention will be further described with respect to endothelial cells genetically engineered with a gene for a therapeutic agent; however, the scope of the invention is not to be limited thereby. For example, the endothelial cells may be genetically engineered with a gene for a protein which is not a therapeutic agent; for example, a marker protein, such as beta-galactosidase.

The endothelial cells are genetically engineered to include a gene for a therapeutic agent by the use of an appropriate vector, with the vector preferably being a retroviral vector. A representative procedure for genetically engineering endothelial cells by the use of a retroviral vector is described in the examples, and such general procedure and others may be employed for introducing other genes into mammalian endothelial cells. Thus, as described in the Examples, the procedure basically involves introduction of an appropriate promoter and DNA for the

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desired therapeutic agent into an appropriate retroviral vector. In addition to the promoter and the gene for the therapeutic agent, other material may be included in the vector such as a selection gene; for example a neomycin resistance gene; a sequence for enhancing expression, etc.

The appropriate vector now containing a gene for at least one desired therapeutic agent is employed for transducing mammalian endothelial cells by procedures generally available in the art.

In accordance with an aspect of the present invention, genetically engineered endothelial cells and in particular those genetically engineered with at least one gene for at least one therapeutic agent may be supported on a solid support. The solid support is preferably one which is biocompatible with blood whereby the solid support including the genetically engineered endothelial cells may be placed in communication with the blood system of a patient. Thus, for example, the solid support may be employed in an extracorporeal device or implanted in a blood vessel (the term implant in a blood vessel includes a by-pass or a shunt for a blood vessel). The implantation may take the form of a blood vessel graft (the term graft includes a shunt or bypass). It is to be understood, however, that the solid support may take a variety of forms, such as pads, strips, gels, etc. and is not limited to grafts.

The genetically engineered mammalian endothelial cells, which include a gene for a therapeutic agent, may be implanted in a blood vessel of a mammal. The mammalian endothelial cells which are genetically engineered in accordance with the present invention are derived from a mammal, and the transformed endothelial cells are implanted in a blood vessel of a mammal of the same species. In a preferred embodiment, the genetically engineered mammalian endothelial cells are implanted in the blood vessel of a host from which the cells were originally derived; i.e., autologous cells are employed. Thus in a preferred

embodiment, endothelial cells are derived from a blood vessel of a patient, genetically engineered to include a gene for at least one therapeutic agent and the genetically engineered cells are implanted in a blood vessel of the patient from which they were derived. In this manner, autologous genetically engineered endothelial cells are employed for in vivo production of a therapeutic agent for treatment of a patient, i.e., gene therapy.

It is to be understood that the genetically engineered endothelial cells may be implanted in a blood vessel on a solid support or implanted directly onto a blood vessel (without the use of a solid support). It is also to be understood that the endothelial cells may be placed on a solid support in an extracorporeal device in communication with the blood system.

In accordance with a preferred embodiment, the genetically engineered mammalian cells are implanted in a blood vessel by providing a blood vessel graft which includes the genetically engineered endothelial cells. The graft now including genetically engineered endothelial cells may be inserted into a blood vessel of a host. Thus, in accordance with one aspect of the present invention, there is provided a blood vessel graft which includes genetically engineered endothelial cells which are suitable for use in a mammalian host, which may be a human or nonhuman mammal.

The blood vessel graft may be any one of a wide variety of vascular grafts and such grafts may be of various sizes. The graft may be used in a vein, an artery, or a capillary. The selection of appropriate grafts is deemed to be within the scope of those skilled in the art from the teachings herein. Although in most cases a synthetic vascular graft is preferred, it is possible within the spirit and scope of the present invention to provide a blood vessel derived from a host with genetically engineered endothelial cells and then graft such blood vessel back into the host. Thus the term graft includes natural and synthetic grafts.

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The graft may be provided with genetically engineered endothelial cells in accordance with the present invention by seeding the genetically engineered endothelial cells onto a suitable blood vessel graft. Representative graft and procedure is disclosed in the Examples. The present invention is not limited to such grafts and procedures. Other grafts and procedures for seeding endothelial cells onto the graft are known in the art and may be used in the present invention. For example, Herring et al Eds. Endothelial Seeding in Vascular Surgery (Grune & Stratton, Inc. Orlando 1987); Ziller et al Eds. Endothelialization of Vascular Grafts, 1st European Workshops on Advanced Technologies in Vascular Surgery, Vienna, Nov. 5-6 (Karger, Basel, 1986). As representative graft materials, there may be mentioned polyesters (for example DACRON); expanded polytetrafluroethylene (Gore-Tex); polyurethanes; coated polyurethanes; such as a silicone coated polyurethane manufactured by Corvita corporation in Miami, Florida; tubular slotted stainless steel stents (Johnson and Johnson) which are coated with a substrate to permit adhesion of the endothelial cells to the stents; natural blood vessels, etc.

The graft, now including genetically engineered endothelial cells, may then be inserted into a blood vessel of a host. The procedures for placing a graft in an appropriate blood vessel are generally known in the art, and such procedures are applicable to the present invention.

Alternatively, endothelial cells may be removed from a blood vessel of a patient, genetically engineered and returned to a blood vessel of the patient, without use of an implantable solid support.

The endothelial cells, which are genetically engineered with an appropriate therapeutic agent, may be genetically engineered in a manner such that the therapeutic agent is secreted into the blood, whereby such therapeutic agent may exert its effect upon cells and tissues either in the immediate vicinity or in more

distal locations. Alternatively, the therapeutic agent may not be secreted from the cells, and exert its effect within or on the genetically engineered endothelial cells upon substances that diffuse into the cell. Thus, for example, adenosine deaminase (ADA) may function within the cell to inactivate adenosine, a toxic metabolite that accumulates in severe combined immunodeficiency syndrome; phenylalanine hydroxylase may function within a cell to inactivate phenylalanine, a toxic metabolite in phenylketonuria, etc.

As hereinabove indicated, the endothelial cells are transformed with a gene for at least one heterologous protein, preferably a therapeutic agent. The term therapeutic agent is used in its broadest sense and means any agent or material which has a beneficial effect on the host. The therapeutic agent may be in the form of one or more proteins. As representative examples, there may be mentioned: CD-4; Factor VIII, Factor LX, von Willebrand Factor, TPA; urokinase; hirudin; the interferons; tumor necrosis factor, the interleukins, hematopoietic growth factors (G-CSF, GM-CSF, IL3 erythropoietin), antibodies, glucocerebrosidase; ADA; phenylalanine hydroxylase, human growth hormone, insulin, etc. The selection of a suitable gene is deemed to be within the scope of those skilled in the art from the teachings herein.

In using the genetically engineered endothelial cells, it is possible to employ a mixture of endothelial cells which includes endothelial cells genetically engineered with a gene for a first therapeutic agent and endothelial cells genetically engineered with a gene for a second therapeutic agent. It is also possible to transform individual endothelial cells with more than one gene.

The genetically engineered endothelial cells may be implanted in a blood vessel alone or in combination with other genetically engineered endothelial cells or with other

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genetically engineered cells, such as smooth muscle cells, fibroblasts, glial cells, keratinocytes, etc.

The use of genetically engineered endothelial cells permits a therapeutic agent to be introduced directly into the blood. As a result of the location of the endothelial cells in immediate contact with the circulating blood, the survival and delivery of a therapeutic agent is facilitated.

The genetically engineered endothelial cells (by selection of high producing clonal populations and/or the use of vectors with enhanced expression) may be employed to produce, in vivo, therapeutically effective amounts of a desired therapeutic agent for treating a patient. In determining the number of cells to be implanted, factors such as the half life of the therapeutic agent; volume of the vascular system; production rate of the therapeutic agent by the cells; and the desired dosage level are considered. The selection of such vectors and cells is dependent on the therapeutic agent and is deemed to be within the scope of those skilled in the art from the teachings herein.

Figures 1a through 1d depict a schematic illustration of vectors used in the present invention.

The following Examples further illustrate the present invention; however, the scope of the invention is not to be limited thereby. In the Examples, unless otherwise specified, restriction enzyme digests, ligations, transformations, etc. may be performed as described in Molecular Cloning, A Laboratory Manual by Maniatis et al.

Example 1

A. To construct the pG2N retroviral vector of the drawing used to genetically engineer endothelial cells to produce rat growth hormone, an SV40 promoted neomycin resistance gene and a rat growth hormone cDNA were placed into the pB2 retroviral vector (Laboratory of Molecular Hematology, NIH). A growth hormone cDNA was obtained by digesting the plasmid RGH-1 (Nature 270, 494 (1977)) with Xho I and Mae III restriction endonucleases

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(Boehringer Mannheim Biochemicals). This rat growth hormone cDNA was eletrophoretically isolated out of an agaorse gel and purified via binding/elution to glass beads, Geneclean (BIO 101, LaJolla, California). This growth hormone cDNA was then blunted using the large fragment of DNA polymerase (Klenow) (New England Biolabs) and nucleotide triphosphates as recommended by the manufacturer. This fragment was then purified with Geneclean.

The B2 vector was constructed in order to replace the Neo^R gene in N2, [M.A. Eglitis, P. Kantoff, E. Gilboa, W.F. Anderson, Science 230, 1395 (1985); D. Armentano et al., J. Virol, 61, 1647 (1987) and shown in the drawing] with a multiple cloning site. N2 was first digested with Eco RI, thereby releasing both the 5' and 3' LTRs with the adjoining MoMLV flanking sequences. The 3' LTR fragment was ligated into the EcoRI site of the plasmid GEM4 (Promega Biotech). The 5' LTR fragment with its flanking gag sequence was then digested with Cla I, Hind III linkers were added, and the fragment was inserted into the Hind III site of pGEM4.

The pB2 vector was digested with the HincII restriction endonuclease (New England Biolabs), and phosphatased using calf alkaline phosphatase. (Boehringer Mannheim Biochemicals). The pB2 plasmid was then purified with Geneclean. The pB2 vector and the rat growth hormone cDNA were then ligated using T4 ligase (New England Biolabs). The ligation was then transformed into competent DH5 bacteria (Bethes da Research Labs). Colonies were then screened for a growth hormone cDNA containing vector. The new vector was called pG2. pG2 was then digested with BamHI (New England Biolabs), purified with Geneclean (Bio 101), and blunt ended with the Klenow fragment (New England Biolabs). A 340 base pair SV40 promoted neomycin resistance gene fragment was isolated from the pSV2CAT plasmid (ATCC accession number 37155) by digesting with PvuII and HindIII (New England Biolabs). This fragment was isolated by agarose gel electrophoresis and purified with Geneclean. The SV40-neomycin resistance fragment was then

ligated using T4 ligase (New England Biolabs) with pG2 and transformed into DH5 competent bacteria per the manufacturers instruction (BRL). Colonies were screened and the resulting plasmid construct was called pG2N.

The SAX vector shown in the drawing was obtained as described in Proc. Natl. Acad. Sci. USA 83:6563 (1986).

The recombinant vectors (N2,SAX, G2N) used in the study were each separately transfected into the currently available retroviral vector packaging cell lines, including the amphotropic packaging lines, PA12 (Science 225:630 (1984) and PA317 (Mol. Cell. Biol 6:2895 (1986), and the ecotropic line, Psi2 (Cell 33:153 (1983). These lines were developed in order to allow the production of helper virus-free retroviral vector particles.

Aortic endothelial cells were obtained from New Zealand White rabbits (2-5 kilograms) by methods described previously for obtaining endothelial cells from bovine pulmonary artery (U.S. Ryan, M. Mortara, C. Whitaker, Tissue & Cell 12, 619 (1980)). The rabbit was anesthetized (1 ml sodium pentobarbital) and the aorta was removed and placed in Hanks buffered saline containing 3X antibiotics. The vessel was slit longitudinally and the luminal surface was scraped with a #11 scalpel blade taking care to scrape each area only once. The initial isolates were grown in Ryan Red medium [Ryan et al J. Tissue Cult. Methods, 10:3 (1986)], purified by selection of endothelial "islands" and passaged with a rubber policeman. Passaged cells were grown in Primaria 25 cm^2 flasks.

A confluent 100 mm tissue culture dish (Costar) was harvested with a cell scraper. Following the dispersal of the cells by titurating 10-20 times with a 5 ml pipet, the cells were plated into 2-100 mm tissue culture dishes with 8 ml Ryan Red medium. After an overnight incubation, the medium was removed and 5 ml retroviral vector supernatant was added with Polybrene at a final concentration of 8 ug/ml. After a 2 hour incubation an additional 5 ml of Ryan Red was added to the dish. The cells

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were incubated overnight and the medium was replaced with 8 ml of Ryan Red. After another overnight incubation G418 was added to a final concentration of 200 ug/ml. The cells were the fed every 3-4 days with Ryan Red containing 200 ug/ml G418. The cell were subsequently maintained in Ryan Red without G418.

G2N-infected RAEC that had been selected in G418-containing growth medium were harvested with a rubber policeman from 2 confluent T75 flasks and suspended in 5 ml of Ryan Red. The cell suspension was titurated 6-7X with a 6 cc syringe and a 23 gauge needle. The cells were pelleted and resuspended in 1.25ml of Ryan Red. A vascular clamp was attached to one of a 10 cm X 4 mm (inner diameter) Corvita graft (Corvita Corp., Miami, FL) which is a silicone coated polyurethane graft. The cell suspension was vortexed and introduced into the open end of the graft with a 3 cc syringe and 20 gauge needle. A second vascular clamp was attached to the open end and the graft was placed into a 50 ml conical tube filled with Ryan Red medium. The conical tube was capped, wrapped in parafilm, and placed into a roller bottle. The roller bottle was rotated overnight at 37 degrees Centrigrade. The next day the clamps were removed and the graft was placed into a 500 ml bottle containing 150 ml of Ryan Red. The bottle was placed in an incubator equilibrated with 5% CO_2, at 37 degrees C. It remained in the incubator and was periodically rotated for the next nine days. At that time the graft was transferred into a T75 flask, fed with fresh medium and periodically sampled for rat growth hormone production over the next four weeks.

rGH continued to be secreted into the tissue culture medium at a rate of approximately 1000 ng/10^6 cells/day for at least 4 weeks after seeding the graft as follows.

Cell on graft 6×10^4 ($cells/cm^2$)	Rat growth hormone production ($ng/10E^6$ cells/24 hours)

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Day 13	930
Day 32	1060

B. Rabbit endothelial cells were also transfected with the vector SAX by the hereinabove described procedure and such tranfected cells were found to express human ADA.

C. The CD4 containing plasmid (pT4B, a gift of Richard Axel of College of Physicians and Surgeons Columbia University, New York, New York) was digested with the restriction endonucleases Eco RI and Bam HI New England Biolabs, Beverly MA) to release the CD4 gene which was isolated by agarose gel electrophoresis followed by purification via binding/elution to glass beads (using the geneclean product, BIO 101, La Jolla CA in the manner recommended by the manufacturer). The CD4 fragment was ligated (using T4 DNA ligase as recommended by the supplier, New England Biolabs) into Eco RI plus Bam HI cut Bluescript cloning vector (stratagene Co. La Jolla CA). The ligation was then transformed into competent DH5 alpha bacteria (Bethesda Research Labs, Gaithersburg MD) and white colonies were isolated and screened for proper insert size to yield the plasmid pCDW. To produce a suitable plasmid based expression vector for the CD4 gene; the plasmid SV2neo (obtained form American Type Culture Collection, Rockville MD) was digested with Hind 3 plus Hpa I, and a synthetic polylinker sequence from the pUC-13 vector (Pharamicia, Piscataway NJ) was inserted (via T4 DNA ligase) in place of the neo gene of pSV2neo. This ligation was transformed into DH5 bacteria (Bethesda Research Labs) and colonies screened for the presence of restriction enzyme sites unique to the polylinker to yield the vector pSVPL. The pSVPL expression vector was further modified by the insertion of an Xho I linker (conditions and reagents supplied by, New England Biolabs) into the Pvu II site on the 5' side of the SV40 early region promoter to produce pSVPLX.

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The pCDW and pSVPLX plasmids were digested with enzymes Hind 3 plus Xba I (New England Biolabs) and their DNAs isolated (using the Gene Clean product) following agarose gel electrophoresis. Ligation of the CD4 fragment into the pSVPLX vector was performed and colonies were screened to yield pSVCDW in which the SV40 virus early region promoter is used to drive the expression of the complete CD4 gene product. The next step was to produce a form of the CD4 gene such that it would be exported from the cell as an extracellular product.

The production of a soluble form of CD4 was accomplished by the use of a specially designed oligonucleotide adaptor to produce a mutant form of the CD4 gene. This adaptor has the unique property that when inserted into the Nhe I site of the CD4 gene it produces the precise premature termination of the CD4 protein amino acid sequence while regenerating the Nhe I site and creating a new Hpa I site. This oligonucleotide adaptor (synthesized by Midland certified reagent Co.) was produced by annealing two phosphorylated oligonucleotides; 1) 5' CTAGCITGAGTGAGIT 3', 2) AACTCACTCAAG and then this product was ligated into the site of pSVCDW. The ligation reaction was then cleaved with Hpa I and then Xho I linkers were added (New England Biolabs). The linker reaction was terminated by heating at 65C for 15 min. and then subjected to digestion with Xho I restriction endonuclease (New England Biolabs). This reaction was then subjected to agarose gel electrophoresis and the fragment containing the SV40-CD_4 adaptor isolated (Geneclean). The retroviral vector N2 was prepared to accept the SV40-CD_4-adaptor fragment by digestion with Xho I and treatment with Calf intestinal phosphatase (Boehringer Mannheim, Indianpolis IN). The ligation of CD4 expression cassette was performed with an insert to vector ratio of 5:1 and then transformed into DH5 competent bacteria (Bethesda Research Labs). Constructs were analyzed by restriction endonuclease digestion to screen for orientation and then grown up in large scale. The

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construct where the SV40 virus promoter is in the same orientation as the viral LTR promoters is known as SSC while the construction in the reverse or reverse orientation is called SCSC.

The SSC vector is packaged into PA317 cell line as described by Miller et al supra. to provide PA 317 cells capable of producing soluble CD4 protein.

The SSC vector packaged PA 317 cells were used to transduce rabbit endothelial cells as hereinabove described.

The transduced endothelial cells were found to express soluble CD-4.

D. Collagen sponges containing adsorbed HBGF-I were surgically implanted in the abdominal cavity of a rat near the liver (Science 241, 1349 (1988). Seven (7) to ten (10) days post implantation, sponges were surgically removed and digested 30 to 60 min. at 37°C with a solution of collagenase in phosphate buffered saline (1 mg/ml) using a tissue culture incubator (5% CO_2). Released cells were collected by centrifugation (10 min., 1000 RPM, 20°C) and washed once with phosphate buffered saline (PBS) and pelleted by centrifugation. Cells were resuspended with 30 ml of media containing:

M199 media (Gibco)
ECGF (crude brain extract) 7.2mg
Heparin (Upjohn) 750 units
20% conditioned cellular media collected as supernatant from confluent dishes (48 hr.) of either bovine aortic or human umbilical vein endothelial cells
10% Fetal Calf serum (Hyclone)
3000 units Penicillan G (Biofluids)
3000 units streptomycin sulfate (Biofluids)

and plated for 16 hours on 100 mm tissue culture disk coated with fibronectin (human) using $1ug/cm^2$. Plated cells were washed with PBS three times and fed 15ml of previously mentioned media.

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Media was changed every 2 days for the duration of the procedures.

Selected rat endothelial cells were transduced with the N-7, SAX, G2N and SSC vectors by the following procedure:

1. 2×10^6 microendothelial cells (monolayer 80% confluent)
2. 2×10^6 viral supernatant
3. Polybrene (8ug/ml)

-Combine 1, 2, 3 in 5ml total volume for 2-3 hours at 37°C (5% CO_2).
-Add 20ml of tissue culture media for 16 hrs. at 37°C (5% CO_2).
-Aspirate off media (virus containing), add fresh culture media.
-After 48-96 hours add G418 (800ug/ml) and culture media.
-Select for one to two weeks changing media every two days.

Cells transduced with N7 expressed neomycin, those transduced with SAX expressed ADA; those transduced with G2N expressed rat growth hormone; and those transduced with SSC expressed sCD4.

The rat endothelial cells transduced with G2N expressed rat growth hormone in vitro as follows:

10^6 cells produce 3.0 µg after 24 hrs.; 6 µg after 48 hrs.; and 9.0 µg after 72 hours.

Example 2

A. Endothelial cells were harvested from segments of adult sheep jugular vein, carotid artery, and femoral vein using the method of Jaffee et al. J.Clin.Invest., 52:2745-2756 (1973). A total of four vessels from three sheep were used. Identification of harvested endothelial cells was confirmed by their cobblestone structure and by confirmed by their cobblestone structure and by binding of the fluorescent ligand DiI-Acetyl-LDL J.Cell.Biol, 99:2034-2040 (1984)). (Biomedical Technologies, Stoughton, Massachusetts). Cells were cultured on fibronectin (Collaborative Research, Bedford, Massachusetts) coated plastic culture dishes (1.0 µg/cm² in M-199 Biofluids, Rockville,

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Maryland) with 20% fetal calf serum (Hyclone Laboratories, Logan, Utah), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Biofluids). Cells were passaged using trypsin-EDTA (Biofluids) digestion. Removal of sheep vessels was done according to protocols approved by the animal use committee of the National Heart, Lung, and Blood Institute.

B. A murine ecotropic packaging line capable of transmitting the ß-galactosidase-containing "BAG" vector (Proc. Natl.Acad Sci, 84:156-160 (1987) was provided by Constance Cepko (Harvard University, Cambridge, Massachusetts). Supernatant from this packaging line was used to generate an amphotropic packaging line from PA-317 cells. A human t-PA cDNA (in plasmid pPA34'f) (J.Biol. Chem, 260 t:11223-11230(1985)) was provided by Sandra Degan (University of Cincinnati, Cincinnati, Ohio). This t-PA cDNA was used, through several subcloning steps, to construct a t-PA containing retroviral vector, B2NSt analogous in construction to the SAX vector. The corresponding plasmid, based on the B2 plasmid, (Science), 343:220-222 (1989)) was transfected into GPE-86 cells, (J.Virol., 62:1120-1124 (1988)) and supernatant from these cells, thereby, generating amphotropic packaging clones capable of transmitting the t-PA gene. Endothelial cells were transduced by incubation for 2 hours with supernatant-containing virions with the retroviral vector, along with 8 µg/ml G-418 for at least 16 days. Duplicate cultures of cells from each vessel harvest were transduced simultaneously with either the t-PA- or ß-galactosidase-containing retroviral vector and, then, cultured, passaged, and selected using identical procedures. In this manner, the t-PA- and ß-galactosidase-transduced cells served as controls for one another in experiments involving either ß-galactosidase activity or t-PA secretion.

C. Tubular slotted stainless steel 1.6-mm diameter stents (Circulation, 76:IV-27 (1987)) (Johnson and Johnson Interventional Systems, Warren, New Jersey) were cut at the

articulation, and each half was seeded with endothelial cells, using a modification of the method of Van der Geissen et al. (J.Intervent. Cardiol, 1:109-120 (1988)). A total of 10 stent segments were seeded. Endothelial cells will not grow on bare metal, and therefore the application of a substrate is necessary before cell seeding. A fibronectin coating is used in vitro to allow endothelial cell adhesion to the stents. Stents were submerged in 100 µg/ml human fibronectin for 15 minutes at 37° and, then, transferred to polypropylene tubes containing a suspension of $6\text{-}10X10^4$ endothelial cells in 0.8 ml culture medium. The tubes were placed in a 37° incubator containing 5% CO_2 and rotated 180° every 10 minutes for 2 hours, after which the stents and cell suspension were placed in wells of plastic tissue-culture dishes and additional culture medium added. Coverage of the stent surfaces was monitored both by phase-contrast microscopy and by incubation of the stents for 4 hours in medium containing DiI-Acetyl-LDL followed by fluorescence microscopy.

D. The presence of the ß-galactosidase gene product was determined by staining with 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) EMBO J., 5:3133-3142 (1986)) of cells either on tissue-culture dishes or in situ on the stents. Levels of human t-PA were determined by enzyme-linked immunosorbent assay (ELISA) on tissue culture supernatants using a commercially available kit (Thromb. Res., 41:527-535 (1986)). (American Diagnostica, New York, New York). Supernatant to be assayed was collected above confluent monolayers in 35-mm dishes, 48 hours after addition of 2 ml fresh medium. For measurement of t-PA secretion from a seeded stent, the stent was transferred to a new well containing fresh medium and, then, began a timed collection of culture medium. Harvested supernatant was centrifuged at 15,000g for 15 minutes to remove cellular debris, made 0.01% with Tween-80, and frozen at -70°C until assayed. The rate of t-PA secretion in nanograms per 10^6

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cells per 24 hours was calculated using a confluent cell density of 3 x 10^4 cells per cm^2 of tissue culture plastic (data not shown).

E. Seeded stents were incubated in medium containing DiI-Acetyl-LDL for 4 hours before expansion. The stents were visualized by fluorescence microscopy to confirm endothelial coverage, and, then, manually placed over a deflated 3.0-mm diameter coronary angioplasty balloon catheter (Scimed Life Systems, Maple Grove, Minnesota). After balloon inflation to 4-6 atmospheres, resulting in complete stent expansion, the balloon was deflated and the stents were removed from the catheters and, again, viewed by fluorescence microscopy.

F. Transduced sheep endothelial cells retained their cobblestone structure and their ability to bind the fluorescent ligand DiI-Acetyl-LDL. No difference in structure was detectable between those cells that had been transduced with the ß-galactosidase vector and those that were transduced with t-PA vector.

G. Only cells in cultures transduced with the ß-galactosidase gene exhibited deep blue cytoplasm on staining with X-Gal. After G-418 selection, most of the ß-galctosidase-transduced cells stained deep blue with X-Gal.

H. Endothelial cells from all four vessels, when transduced with the t-PA vector, secreted immunoreactive t-PA. Rates of t-PA secretion (mean ± SD of duplicate tissue culture wells, expressed as ng/10^4 cells/24 hours) were femoral vein, 370 ± 8; carotid artery, 660 ± 240; jugular vein 1, 230 ± 6; jugular vein 2, 200 ± 18. t-PA production by the ß-galactosidase- transduced cells was below the lower limit of sensitivity of the assay (i.e., less than 5 ng/10^4 cells/24 hours) in all of the supernatants tested.

I. Fluorescence microscopy of six of the seeded stents confirmed complete coverage of the visible stent surfaces. When eight stents seeded with either ß-galactosidase- or

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t-PA-transduced endothelial cells were stained with X-Gal, the stents covered with ß-galactosidase- carrying cells turned blue, whereas the stents covered with t-PA-secreting cells did not. Measurement of human t-PA levels from the cell culture medicum surrounding the stents confirmed that t-PA was being secreted only by the t-PA-transduced endothelial cells. Three stents seeded with t-PA-transduced endothelial cells, secreted 6.3, 4.8, and 2.6 ng t-PA/24 hours. t-PA secretion by the ß-galactosidase-transduced cells on each of three stents, if present, was below the limit of detection of the assay. To check the internal consistency of our results, the measured t-PA secretion from each of three lines of transduced cells was used both before and after they were seeded onto stents to calculate the surface area of the stents. This calculation is based on the assumption that the density of the cells and the rate of t-PA secretion do not change when the cells are on the stents. A stent surface area (mean ± SD) of 48 ± 19 mm was calculated, not significantly different from the manufacturer's value of 42 mm^2 (personal communication, Johnson and Johnson Interventional Systems, Warren, New Jersey).

J. Four stents covered with DiI-Acetyl-LDL- stained endothelial cells were expanded using balloon catheters and immediately viewed with a fluorescene microscope. Near-complete retention of the cells on the exterior surfaces of all four stents was confirmed. X-Gal staining of stents was confirmed. X-Gal staining of stents carrying ß-galactosidase- transduced cells permitted evaluation of cellular retention on all surfaces after balloon inflation. The stents were viewed with a dissecting microscope, and cellular retention on all surfaces was estimated. A total of eight expanded stents were observed after X-Gal staining, four covered with ß-galactosidase- transduced endothelial cells. Much of the interior lumen surface of the stents was free of cells after balloon inflation but that the

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cellular layer on the exterior and lateral stent-strut surfaces was largely intact.

Although the scope of the present invention is not intended to be limited to any theoretical reasoning, it is believed that an intravascular stent seeded with endothelial cells as hereinabove described may produce a local thrombolytic environment in vivo. High level secretion of t-PA adjacent to a forming clot may permit t-PA to be concentrated through the high affinity binding of t-PA to fibrin. (Thorsen, et al., Thromb D. Haemorrh, 28:65-74 (1972)). In this manner, fibrinolytic activity would be directed to microthrombi beginning to form on the stent surface or downstream, thus preventing the formation of occlusive thrombi. It has been demonstrated (Hergreuter, et al., Plast. Reconstr. Surg., 81:418-424 (1988)) in a rabbit model that locally administered t-PA could abort thrombus formation on a highly thrombogenic inverted artery. Intravascular stents are far less thrombogenic than is an inverted vessel, and it is possible that localized delivery of nanogram quantities of t-PA will result in sufficient thrombolytic activity to prevent stent-related thrombotic events.

It has also been theorized that the implantation of genetically engineered endothelial cells on stent surfaces offers a potential means of preventing intimal hyperplasia because implanted endothelial cells would be in direct contact with the intima and could be engineered to secrete proteins capable of inhibiting intimal growth.

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

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WHAT IS CLAIMED IS:

1. A product, comprising:

endothelial cells genetically engineered with at least one gene for at least one heterologous protein.

2. The product of claim 1 wherein the cells are human endothelial cells.

3. The product of claim 1 wherein the endothelial cells are microvascular endothelial cells.

4. The product of claim 1 wherein the endothelial cells are macrovascular endothelial cells.

5. The product of claim 1 wherein the endothelial cells are genetically engineered with a retroviral vector including a gene for a heterologous protein.

6. The product of claim 5 wherein the heterologous protein is a therapeutic agent.

7. The product of claim 1 wherein the endothelial cells are mammalian endothelial cells.

8. The product of claim 1 wherein the heterologous protein is secretable from the cells.

9. A product comprising:

a solid support, said solid support including endothelial cells genetically engineered with at least one gene for at least one heterologous protein.

10. The product of claim 9 wherein the solid support is compatible with blood.

11. The product of claim 10 wherein the solid support is a vascular graft.

12. The product of claim 11 wherein the graft is a synthetic graft.

13. The product of claim 10 wherein the solid support is a tubular slotted stainless steel intravascular stent, said stent being coated with a substrate for permitting adhesion of said endothelial cells to said stent.

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14. The product of claim 11 wherein the cells are human endothelial cells.

15. The product of claim 11 wherein the endothelial cells are microvascular endothelial cells.

16. The product of claim 11 wherein the endothelial cells are macrovascular endothelial cells.

17. The product of claim 11 wherein the endothelial cells are genetically engineered with a retroviral vector including a gene for a heterologous protein.

18. The product of claim 12 wherein the heterologous protein is a therapeutic agent.

19. The product of claim 18 wherein the heterologous protein is secretable from the cells.

20. A process for gene therapy, comprising:

implanting in a blood vessel of a host endothelial cells genetically engineered with at least one gene for a heterologous protein which is a therapeutic agent for the host.

21. The process of claim 20 wherein said endothelial cells are implanted by implanting in a blood vessel of the host a biocompatible solid support containing genetically engineered endothelial cells.

22. The process of claim 21 wherein the host is a human and the genetically engineered endothelial cells are human endothelial cells.

23. The process of claim 22 wherein the endothelial cells are genetically engineered with a retroviral vector including a gene for the therapeutic agent.

24. The process of claim 21 wherein the host is a human patient and the endothelial cells are autologous endothelial cells.

25. The process of claim 24 wherein the solid support is a vascular graft.

26. The process of claim 25 wherein the endothelial cells are genetically engineered with a retroviral vector including a gene for the therapeutic agent.

27. The process of claim 26 wherein the therapeutic agent is secretable from the cells.

28. The process of claim 20 wherein said endothelial cells are comprised of a first portion genetically engineered to express a first therapeutic agent and a second portion genetically engineered to express a second therapeutic agent different from the first therapeutic agent.

29. The product of claim 7 wherein said gene encodes for soluble CD-4.

30. The product of claim 7 wherein said gene encodes for ADA.

31. The product of claim 7 wherein said gene encodes for TPA.

32. The product of claim 10 wherein the gene encodes for a member selected from the group consisting of soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, the interferons, tumor necrosis factor, the interleukins, hematopoietic growth factors, antibodies, glucocerebrosidase, ADA, phenylalanine hydroxylase, human growth hormone, and insulin.

33. The process of claim 24 wherein the gene encodes for a member selected from the group consisting of soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, the interferons, tumor necrosis factor, the interleukins, hematopoietic growth factors, antibodies, glucocerebrosidase, ADA, phenylalanine hydroxylase, human growth hormone, insulin and erythropoietin.

34. The product of claim 10 wherein the gene encodes for soluble CD-4.

35. The product of claim 10 wherein the gene encodes for ADA.

36. The product of claim 10 wherein the gene encodes for TPA.

37. The process of claim 33 wherein the gene encodes for soluble CD-4.

38. The process of claim 33 wherein the gene encodes for ADA.

39. The process of claim 33 wherein the gene encodes for TPA.

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FIG. 1a

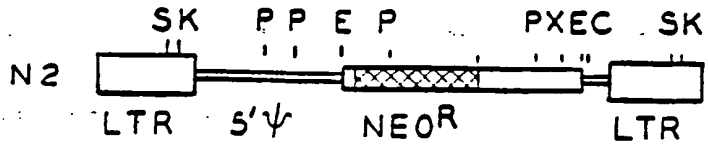


FIG. 1b

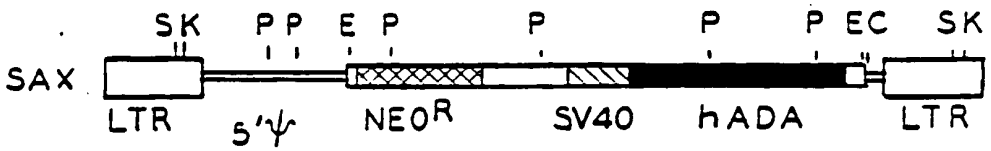


FIG. 1c

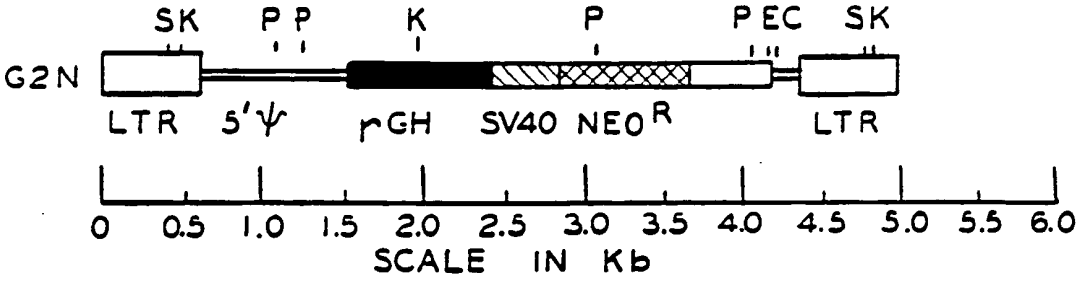
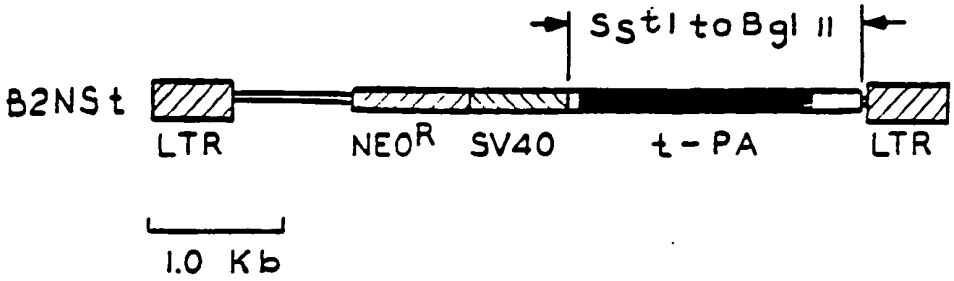


FIG. 1d



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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05575

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00, C12N 5/08, C07G 15/00, C07K 7/00; A61K 48/00; A61D 2/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL.	435/172.2, 173.2, 240.23, 240.243, 268, 269 935/48, 52, 53, 62 623/1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Weinberg et al Science 231: 397-400. 1 January 1986 (See the entire document)	9-12, 14-28 32-39
Y	Yeager et al ASAIO Transactions 34: 88-94. 1988 (Entire document)	19-28,33. 33-39
Y	Williams et al. Nature 310: 476-480. 1984 (Entire document)	1-8, 20-31, 33-39
Y	Seldon et al. Science 236: 714-718. 1987 (Entire document)	1-8,14-31, 33, 37-39
Y	Anderson. Science 226: 401-409. 1984 (Entire document)	1-8, 20-31, 33, 37-39
Y	Coplan et al U.S, A, 4,609,551 02 September 1986	13
X	Vander Giessen et al. Journal of International Cardiology. Vol. 1(2): 109-120. 1988	13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5 March 1990	20 APR 1990
International Searching Authority: ISA/US	Signature of Authorized Officer: Suzanne Ziska for

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

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